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Induction of Disease by a Molecularly Cloned Highly Pathogenic Simian Immunodeficiency Virus/Human Immunodeficiency Virus Chimera Is Multigenic

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One of three full-length infectious molecular clones of $SHIV_{DH12R}$, designated $SHIV_{DH12R-CL-7}$ and obtained from productively infected rhesus monkey peripheral blood mononuclear cells, directed rapid and irreversible loss of $CD4^+$ T cells within 3 weeks of its inoculation into Indian rhesus monkeys. Induction of complete $CD4^+$ T-cell depletion by $SHIV_{DH12R-CL-7}$ was found to be dependent on inoculum size. The acquisition of this pathogenic phenotype was accompanied by the introduction of 42 amino acid substitutions into multiple genes of parental nonpathogenic $SHIV_{DH12}$. Transfer of the entire $SHIV_{DH12R-CL-7}$ *env* gene into the genetic background of nonpathogenic $SHIV_{DH12}$ failed to confer the rapid $CD4^+$ T-lymphocyte-depleting syndrome; similarly, the substitution of *gag* plus *pol* sequences from SIV_{smE543} for analogous SIV_{mac239} genes in $SHIV_{DH12R-CL-7}$ attenuated the pathogenic phenotype. Amino acid changes affecting multiple viral genes are necessary, but insufficient by themselves, to confer the prototypically rapid and irreversible $CD4^+$ T-cell-depleting phenotype exhibited by molecularly cloned $SHIV_{DH12R-CL-7}$.

Simian immunodeficiency virus (SIV)/human immunodeficiency virus (HIV) chimeras (SHIVs) have proven to be useful reagents for studies of primate lentivirus pathogenesis and vaccine development (2, 3, 10, 21). Because they bear the HIV type 1 (HIV-1) envelope glycoprotein, SHIVs have been valuable for assessing vaccine-induced anti-HIV-1 neutralizing antibodies (2, 3, 5, 18, 24). Furthermore, highly pathogenic SHIVs induce an unusually rapid, complete, and irreversible depletion of $CD4^+$ T lymphocytes in rhesus monkeys, thereby providing a readily demonstrable endpoint in vaccine experiments. Some highly pathogenic SHIVs were initially isolated from rhesus monkeys following multiple animal-to-animal passages of nonpathogenic viruses (14, 17). We previously reported that highly pathogenic $SHIV_{DH12R}$ emerged during a single in vivo passage in a rhesus monkey treated with an anti-human CD8 monoclonal antibody at the time of its primary infection with nonpathogenic $SHIV_{DH12}$ (11). Because the intrinsic genetic heterogeneity of available uncloned highly pathogenic SHIV stocks could be contributing to the inconsistent disease phenotypes observed in some vaccine experiments, a molecularly cloned SHIV, capable of replicating to high levels in vitro and in vivo and consistently inducing rapid and complete loss of $CD4^+$ T cells in inoculated Indian rhesus monkeys, was constructed.

Cloning strategy. Lambda phage vectors have previously been used to obtain full-length molecular clones of unintegrated HIV-1 DNA from productively infected cells (1, 6, 22). The identification of a restriction enzyme that cuts the viral DNA a single time, thereby permitting the cloning of one and two long terminal repeat circular DNA molecules produced in newly infected cells is an initial step in this process. Following transfer to plasmids, cloned circularly permuted DNA molecules can be readily converted to linear, correctly oriented forms of retroviral DNA (16, 22).

In preliminary experiments to generate molecular clones of highly pathogenic $SHIV_{DH12R}$ (11), DNA was prepared from rhesus monkey peripheral blood mononuclear cells (PBMC), infected with the $SHIV_{DH12R-PS1}$ derivative (24), and subjected to digestion by several restriction enzymes. Southern blot hybridization analyses revealed that EcoRI converted the circular forms of viral DNA into linear molecules approximately 10 kbp in size, consistent with the presence of a single EcoRI site within unintegrated $SHIV_{DH12R}$ DNA (data not shown). Accordingly, Hirt-fractionated (9), $SHIV_{DH12R-PS1}$-infected monkey PBMC DNA was digested with EcoRI and ligated to similarly cleaved lambda phage EMBL-4 DNA (Stratagene/Biocrest; Cedar Creek, Tex.) as previously described (22). Positive recombinant phage plaques, identified by using the 8.1-kbp HaeII-XhoI fragment (encompassing the *gag* through the *env* sequences) from $SHIV_{DH12}$ (20) as a probe, were expanded, and the insert was transferred to pBR322. Two long terminal repeat linear forms of SHIV DNA were reconstituted from SalI-EcoRI and EcoRI-NarI subfragments of each clone as previously described (16, 22). Three of the full-length $SHIV_{DH12R}$ clones ($SHIV_{DH12R-CL-7}$, $SHIV_{DH12R-CL-8}$, and $SHIV_{DH12R-CL-10}$) obtained generated progeny virions following transfection of HeLa cells, each of which exhibited robust and indistinguishable infection kinetics in rhesus monkey PBMC (data not shown). During these spreading infections, the three cloned SHIVs were highly cytopathic, inducing large syncytia similar to those observed with parental uncloned $SHIV_{DH12R}$.

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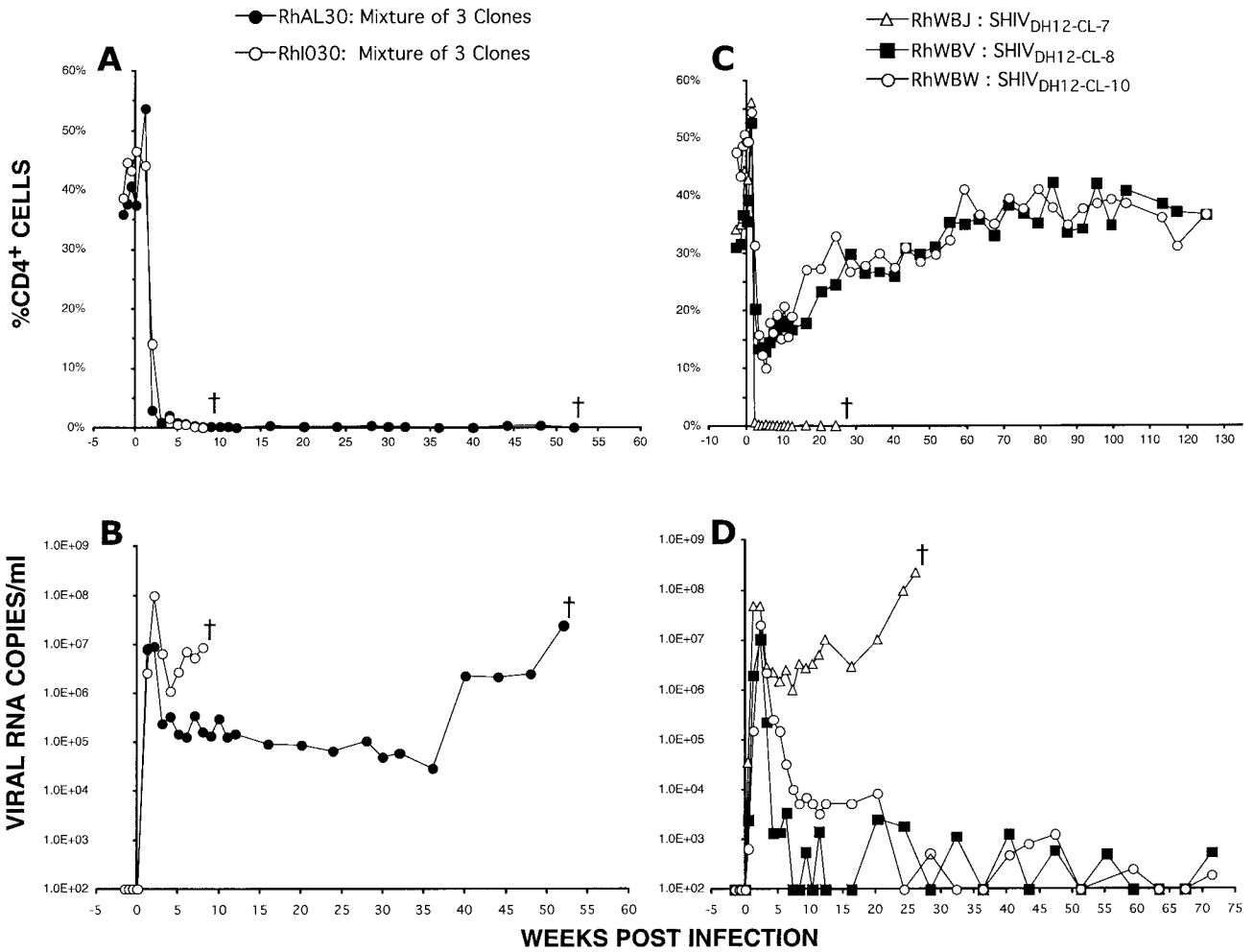

FIG. 1. One of three $SHIV_{DH12R}$ molecular clones induces rapid and complete depletion of $CD4^+$ T cells in rhesus monkeys. Rhesus macaques RhAL30 and RhIO30 were inoculated intravenously (300 μl) with a mixture of undiluted supernatants from transfected HeLa cells, and levels of $CD4^+$ T lymphocytes (A) and plasma viral RNA (B) were determined as previously described (7, 10, 11, 19). Animals RhWBJ, RhWBV, and RhWBW were individually inoculated with $SHIV_{DH12R\text{-}CL\text{-}7}$ (3×10^4 $TCID_{50}$), $SHIV_{DH12R\text{-}CL\text{-}8}$ (1.6×10^4 $TCID_{50}$), and $SHIV_{DH12R\text{-}CL10}$ (2.5×10^4 $TCID_{50}$), respectively. The resulting $CD4^+$ T-cell levels (C) and plasma viremia (D) were determined.

Pathogenicity of cloned SHIVs in rhesus monkeys. In the initial test of pathogenicity, 5 ml of HeLa cell supernatant from each of the three cloned SHIV transfections was subjected to ultracentrifugation ($1.5 \times 10^5 \times g$ for 30 min) and the pelleted virions were resuspended in 500 μl of Dulbecco's minimal essential medium. Equal volumes (250 μl) of the suspended virus preparations were combined, and 300 μl of this mixture was inoculated intravenously into two rhesus monkeys. As shown in Fig. 1A and B, animals RhAL30 and RhI030 rapidly lost $CD4^+$ T cells, reaching levels of less than 20 cells/μl of plasma by week 3, generated high levels of peak and postpeak plasma viremia, and were euthanized at weeks 8 and 57 because of intractable diarrhea and marked weight loss, respectively. This result indicated that one or more of the three $SHIV_{DH12R}$ molecular clones conferred the highly pathogenic phenotype. In a follow-up experiment, virus stocks of each clone were prepared in rhesus monkey PBMC as previously described (13) and high doses of each were inoculated individually into three different macaques. Figure 1C shows that only the recipient of $SHIV_{DH12R\text{-}CL\text{-}7}$ (inoculum size, 3×10^4 50% tissue culture infective doses [$TCID_{50}$]) experienced the complete depletion of $CD4^+$ T lymphocytes (8 $CD4^+$ T cells/μl of plasma at week 3) typically observed with parental uncloned $SHIV_{DH12R}$ (7, 10). The two monkeys inoculated with $SHIV_{DH12R\text{-}CL\text{-}8}$ or $SHIV_{DH12R\text{-}CL\text{-}10}$ (receiving 1.6×10^4 and 2.5×10^4 $TCID_{50}$, respectively) had marked but transient declines (to 170 and 113 cells/μl of plasma at week 3, respectively) of their $CD4^+$ T cells, which gradually returned to preinoculation levels (929 and 1,139 cells/μl of plasma at week 120, respectively). While all three virus-inoculated animals experienced high peak plasma virus loads ($>10^7$ RNA copies/ml) at weeks 2 to 3 postinfection, only monkey RhWBJ, which received $SHIV_{DH12R\text{-}CL\text{-}7}$, continued to produce high and sustained postpeak levels of viral RNA (Fig. 1D). Animal RhWBJ was euthanized at week 26 with severe anorexia and marked weight loss.

We previously reported that the rapid and irreversible loss of $CD4^+$ T lymphocytes induced by uncloned $SHIV_{DH12R}$ was dose dependent (7, 12). This unusual phenotype could reflect the effect of a small subpopulation of highly pathogenic virions,

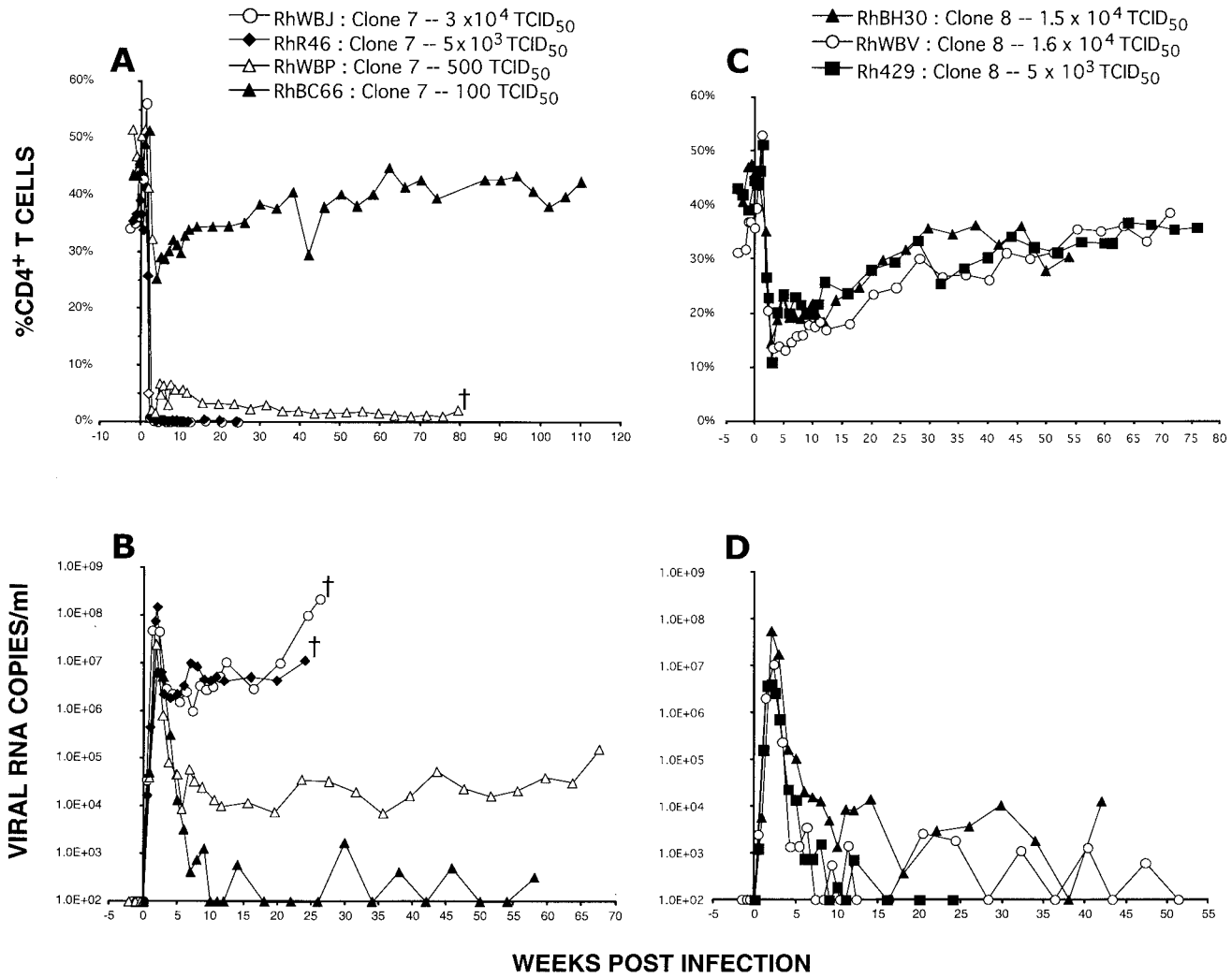

FIG. 2. The rapid and complete loss of rhesus monkey $CD4^+$ T cells induced by $SHIV_{DH12R\text{-}CL\text{-}7}$ is inoculum size dependent. Monkeys RhWBJ, RhR46, RhWBP, and RhBC66 were inoculated intravenously with the indicated amounts of $SHIV_{DH12R\text{-}CL\text{-}7}$, and the levels of $CD4^+$ T lymphocytes (A) and plasma viral RNA (B) were determined. Monkeys RhBH30, RhWBV, and Rh429 were inoculated intravenously with the indicated amounts of $SHIV_{DH12R\text{-}CL\text{-}8}$, and the levels of $CD4^+$ T lymphocytes (C) and plasma viral RNA (D) were determined.

present in the uncloned virus stock, which was solely responsible for the rapid and complete loss of $CD4^+$ T cells in animals inoculated with larger amounts of virus (e.g., 625 $TCID_{50}$ or greater [7]). To ascertain whether this dose-dependent phenotype was also an intrinsic property of molecularly cloned $SHIV_{DH12R\text{-}CL\text{-}7}$, four monkeys were inoculated with decreasing amounts of $SHIV_{DH12R\text{-}CL\text{-}7}$. As can be seen in Fig. 2A, the two macaques receiving 30,000 and 5,000 $TCID_{50}$ of $SHIV_{DH12R\text{-}CL\text{-}7}$ both sustained rapid declines of their $CD4^+$ T cells and were euthanized at weeks 25 and 26 because of diarrhea and marked weight loss, a clinical outcome that was accompanied by high plasma virus loads (Fig. 2B). Three other macaques inoculated with 5,000 $TCID_{50}$ also experienced complete depletion of $CD4^+$ T lymphocytes within weeks of inoculation and were sacrificed between weeks 13 and 25 because of their deteriorating clinical condition (data not shown). The systemic depletion of $CD4^+$ T lymphocytes by $SHIV_{DH12R\text{-}CL\text{-}7}$ was similar histopathologically to that previously described for the uncloned parental $SHIV_{DH12R}$ virus stock (10). $CD4^+$ T-cell loss from peripheral and internal (mesenteric and colonic) lymph nodes began by day 10 postinoculation and predominantly affected lymphocytes in paracortical regions; by day 21 postinfection, an 80 to 90% reduction of this T-cell subset was observed in multiple lymph node specimens. Virus-producing cells peaked on day 10 in the T-cell-rich regions of lymph nodes and on day 14 in the thymic medulla, coincident with the observed slightly delayed loss of $CD4^+$ T cells from the thymus. Postmortem examination revealed diffuse lymphoid depletion, marked thymic atrophy, and evidence of cryptosporidiosis and disseminated candidiasis in several of the $SHIV_{DH12R\text{-}CL\text{-}7}$-infected monkeys.

Rapid and severe loss of $CD4^+$ T cells (51 cells/μl of plasma at week 3) also occurred in the animal (RhWBP) inoculated with 500 $TCID_{50}$ of $SHIV_{DH12R\text{-}CL\text{-}7}$ (Fig. 2A), but in this case, the depletion did not reach baseline levels (<1% of $CD4^+$ T lymphocytes, <20 cells/mm^3) until week 63 postinfection. Monkey WBP had to be euthanized at week 79 because of anorexia and intractable diarrhea. Finally, the inoculation of

Amino Acid Changes $SHIV_{DH12}$-->$SHIV_{DH12R-CL-7}$ (Position)	
Gag	V-->A gag (3)
	N-->Y gag (5)
	L-->F gag (372)
	D-->E gag (498)
Pol	F-->V pol (107)
	D-->E pol (225)
	S-->L pol (413)
	S-->L pol (736)
	I-->V pol (755)
	A-->V pol (821)
Vpx	M-->V vpx (81)
Vpr	P-->S vpr (36)
Rev	R-->K rev (17)
	D-->E rev (69)
	A-->P rev (70)
	V-->A rev (97)
Tat	S-->P tat (75)
Vpu	I-->T vpu (18)

Amino Acid Changes $SHIV_{DH12}$-->$SHIV_{DH12R-CL-7}$ (Position)	
D-->N env (132)	gp120
K-->E env (144)	
G-->E env (152)	
D-->N env (167)	
D-->N env (185)	
N-->S env (187)	
F-->L env (277)	
V-->A env (345)	
S-->N env (373)	
F-->S env (714)	gp41
V-->A env (742)	
T-->S env (755)	
W-->R env (783)	
L-->W env (789)	
T-->I env (811)	
A-->T env (847)	
M-->R nef (7)	Nef
Y-->R nef (17)	
E-->G nef (75)	
R-->G nef (77)	
T-->A nef (110)	
S-->G nef (112)	
E-->K nef (191)	
R-->K nef (245)	

	Clone 7	Clone 8
Vpr (94)	A	T
Env gp41 (771)	V	A
Env gp41 (811)	I	T
Env gp41 (823)	I	V
Nef (7)	R	L
Nef (110)	A	T
Nef (112)	G	S
Nef (122)	F	Y
Nef (245)	K	R

FIG. 3. Amino acid changes associated with the acquisition of a highly pathogenic phenotype. The 42 amino acid differences between nonpathogenic parental $SHIV_{DH12}$ and highly pathogenic $SHIV_{DH12R-CL-7}$ (top) and between nonpathogenic $SHIV_{DH12R-CL-8}$ and highly pathogenic $SHIV_{DH12R-CL-7}$ (bottom) are shown. Note that some of the differences between $SHIV_{DH12R-CL-8}$ and $SHIV_{DH12R-CL-7}$ are not listed in the top panel because they represent amino acid substitutions present in $SHIV_{DH12R-CL-8}$ and not in $SHIV_{DH12R-CL-7}$.

macaque RhBC66 with 100 $TCID_{50}$ of $SHIV_{DH12R-CL-7}$ resulted in only a modest and transient $CD4^+$ T-cell decline (557 cells/μl of plasma at week 5), with a return to preinfection levels by week 40 (1,070 cells/μl of plasma) (Fig. 2A). This moderate $CD4^+$ T-lymphocyte depletion was nonetheless associated with a high peak plasma virus load (6.3×10^6 viral RNA copies/ml) but low to undetectable postpeak viremia (Fig. 2B). Thus, as was the case for uncloned $SHIV_{DH12R}$, the clinical outcome of $SHIV_{DH12R-CL-7}$ infection was also dependent on inoculum size, indicating that this was an intrinsic property of both the uncloned and cloned viruses.

To be certain that the partial and transient depletion of $CD4^+$ T lymphocytes induced by $SHIV_{DH12R-CL-8}$ in monkey RHWBV (Fig. 1C) was an intrinsic property of this clone and did not represent an idiosyncratic animal-specific response to virus, two additional animals were inoculated with high doses of $SHIV_{DH12R-CL-8}$. In both cases, marked but incomplete loss of $CD4^+$ T cells occurred during the first weeks of infection (to 270 cells/μl [RhBH30] and 170 cells/μl [Rh429] at week 3 [Fig. 2C]); this depletion was associated with prompt control of plasma viremia (Fig. 2D). The patterns of $CD4^+$ T-lymphocyte depletion and restoration in the three $SHIV_{DH12R-CL-8}$-infected monkeys were similar to one another and clearly distinguishable from that observed with the animals inoculated with $SHIV_{DH12R-CL-7}$. It is also worth noting that $SHIV_{DH12R-CL-7}$ and $SHIV_{DH12R-CL-8}$ exhibited indistinguishable infection kinetics and cytopathicity following infection of cultured PBMC (data not shown).

Determinants of SHIV pathogenicity. Complete nucleotide sequencing of $SHIV_{DH12R-CL-7}$ revealed that 42 amino acid changes, relative to the starting sequence of nonpathogenic $SHIV_{DH12}$ (previously designated $SHIV_{MD14YE}$ [20]), had accompanied the acquisition of the highly pathogenic phenotype (Fig. 3, top). Although these changes were distributed throughout the viral genome, they primarily affected the *env* and *nef* genes. On the basis of increased chemokine receptor binding affinity, membrane fusion capacity, and/or neutralization resistance, earlier analyses of the molecularly cloned $SHIV_{KB9}$ derivative of $SHIV_{89.6P}$ (15) and the molecularly cloned $SHIV_{HXBc2P-3.2}$ derivative of $SHIV_{KU-1}$ (4) concluded that

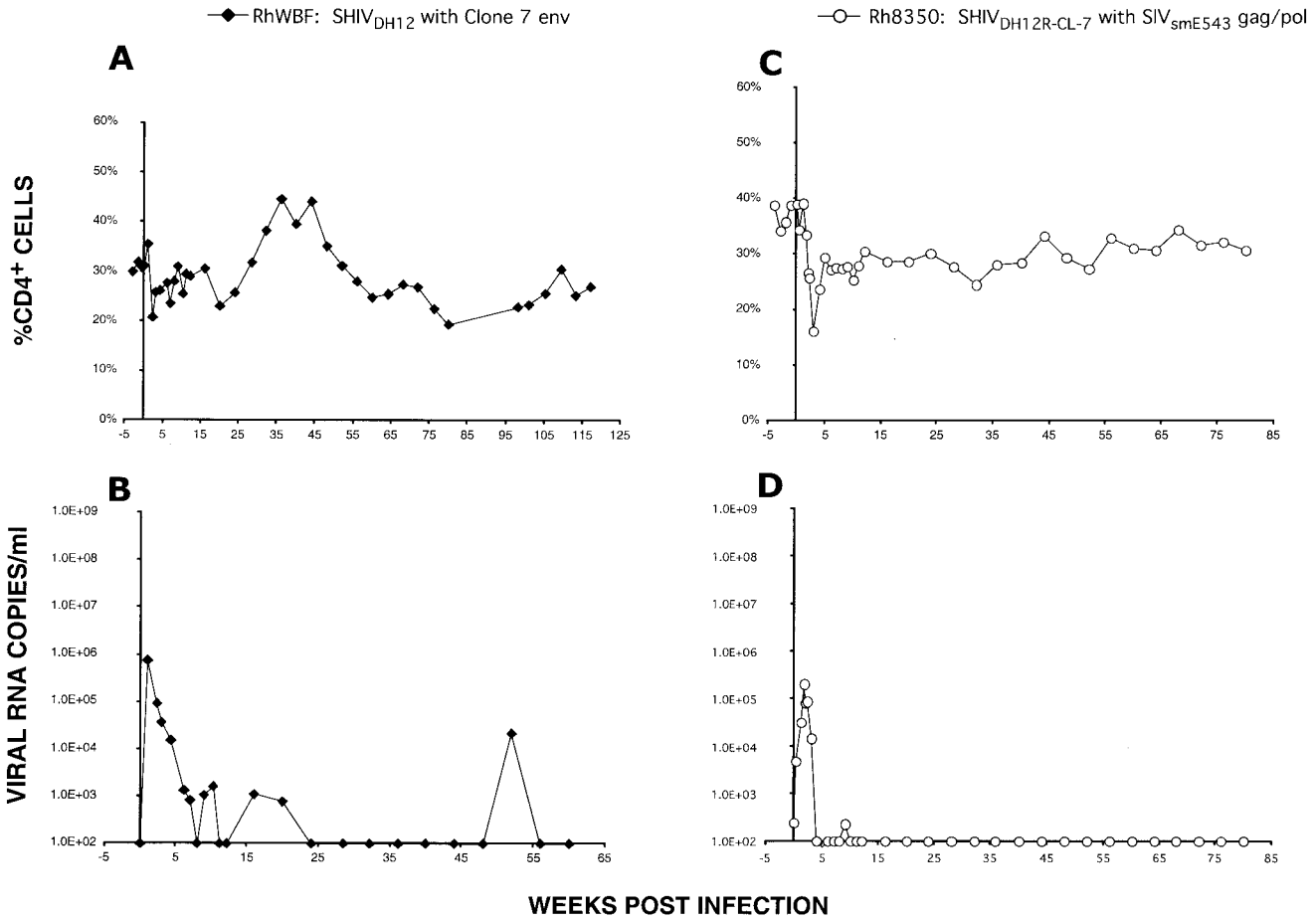

FIG. 4. Altered *gag*, *pol*, and *env* sequences in SHIV$_{DH12R-CL-7}$ are necessary but not sufficient to confer the highly pathogenic phenotype. Macaque RhWBF was inoculated intravenously with SHIV$_{DH12[gp160-CL-7]}$ (75,000 TCID$_{50}$), in which only the *env* gene from SHIV$_{DH12R-CL-7}$ was inserted into nonpathogenic SHIV$_{DH12}$ (A and B). Animal Rh8350 was inoculated with SHIV$_{DH12R-E543(GPV)}$ (16,000 TCID$_{50}$), in which the 5-kbp NarI-BstBI fragment bearing *gag*, *pol*, and *vif* sequences from SIV$_{smE543}$ was introduced into SHIV$_{DH12R-CL-7}$ (C and D). Plasma viremia and CD4$^+$ T-cell levels were determined as indicated.

changes in the HIV-1 envelope glycoprotein component of highly pathogenic SHIVs were the primary determinant for inducing the rapid loss of CD4$^+$ T lymphocytes in vivo. To ascertain whether the 17 amino acid substitutions in SHIV$_{DH12R-CL-7}$ Env, by themselves, could confer the rapid and irreversible CD4$^+$ T-cell-depleting phenotype, the 2.7-kbp HindIII fragment (positions 6333 to 9079) encoding the entire *env* gene of SHIV$_{DH12R-CL-7}$ was transferred into the background of original nonpathogenic strain SHIV$_{DH12}$. A stock of the resultant chimera (SHIV$_{DH12[gp160-CL-7]}$) was prepared in rhesus monkey PBMC and used to inoculate macaque RhWBF with 75,000 TCID$_{50}$. As shown in Fig. 4A, SHIV$_{DH12[gp160-CL-7]}$ did not induce the signature rapid loss of CD4$^+$ T lymphocytes, replicating to only modest levels (peak plasma viremia of 7.5×10^5 RNA copies/ml) during the initial weeks of infection (Fig. 4B). The inability of the SHIV$_{DH12R-CL-7}$ *env* gene to confer the unique pathogenic phenotype was also consistent with nucleotide sequence analyses of the pathogenic SHIV$_{DH12R-CL-7}$ and nonpathogenic SHIV$_{DH12R-CL-8}$ genomes, which indicated that both viruses have identical gp120s at both the nucleotide and amino acid levels (Fig. 3, bottom). More importantly, the comparison of the two viruses revealed that the distinctive CD4$^+$ T-lymphocyte-depleting properties of SHIV$_{DH12R-CL-7}$ versus SHIV$_{DH12R-CL-8}$ are due to sequences encoding Nef, gp41, and Vpr; no changes affecting *cis*-acting elements such as the Rev responsive element, transcriptional regulatory sequences, or packaging signals were noted.

The amino acid comparison of SHIV$_{DH12R-CL-7}$ with its nonpathogenic parent, SHIV$_{DH12}$, shown in Fig. 3 (top) indicates that the acquisition of a more aggressive pathogenic phenotype was accompanied by 10 amino acid substitutions mapping to the SIV$_{mac239}$ *gag* and *pol* genes present in SHIV$_{DH12R-CL-7}$. The contribution of these SIV changes was evaluated by substituting the 5,041-bp NarI-BstBI fragment, carrying *gag*, *pol*, and *vif* sequences, from pathogenic SIV$_{smE543}$ (8) for analogous SIV$_{mac239}$ sequences in SHIV$_{DH12R-CL-7}$. The molecular clone, designated SHIV$_{DH12R-E543(GPV)}$, was transfected into HeLa cells, and the culture supernatant was used to prepare a virus stock in rhesus monkey PBMC. Despite receiving 16,000 TCID$_{50}$ of SHIV$_{DH12R-E543(GPV)}$ intravenously, monkey Rh8350 experienced only a modest loss of CD4$^+$ T cells (248 cells/μl of plasma at week 2) and a relatively low peak plasma viremia (5×10^5 viral RNA copies/ml) (Fig. 4C and

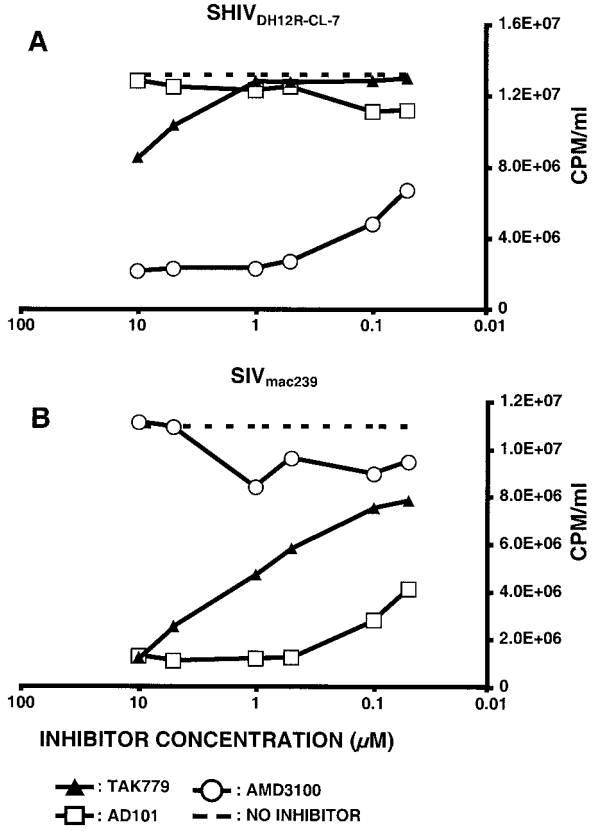

FIG. 5. Coreceptor usage of SHIV$_{DH12R-CL-7}$ and SIV$_{mac239}$ for entry into macaque PBMC. SHIV$_{DH12R-CL-7}$ and SIV$_{mac239}$ were spinoculated onto rhesus PBMC in the presence of the indicated small-molecule coreceptor inhibitors. Rhesus monkey PBMC (5×10^4 cells) were dispensed into 96-well round-bottom plates. The inhibitor concentrations used were 0.05, 0.1, 0.5, 1.0, 5.0, and 10 μM. Reverse transcriptase activity released into the medium on day 5 postinfection was determined in the absence (dashed line) or presence of inhibitor.

D). This attenuated in vivo response very likely reflects differences between the *gag* and *pol* genes of SIV$_{smE543}$ and SIV$_{mac239}$, as well as differences between SIV$_{smE543}$ and the 10 amino acid changes introduced into the *gag* and *pol* sequences attending the evolution of SHIV$_{DH12R-CL-7}$ from its nonpathogenic SHIV$_{DH12}$ parent. Taken together, these results indicate that changes affecting *gag-pol* and *env* are necessary, but insufficient by themselves, to confer the prototypically rapid and irreversible disease phenotype exhibited by SHIV$_{DH12R-CL-7}$.

SHIV$_{DH12R-CL-7}$ uses CXCR4 for entry into rhesus monkey PBMC. The *env* gene of nonpathogenic SHIV$_{DH12}$ was derived from HIV-1$_{DH12}$ (19), previously reported to use CXCR4 and CCR5 for entry into the human glioblastoma cell line U87MG expressing CD4 (27). To assess the chemokine receptor used by SHIV$_{DH12R-CL-7}$ for replication in rhesus monkey PBMC, cells were infected in the presence of small-molecule coreceptor-targeted inhibitors specific for CCR5 or CXCR4. The production of progeny virus was measured as reverse transcriptase activity released into the medium on day 5 postinfection. As shown in Fig. 5A, infection of monkey PBMC by SHIV$_{DH12R-CL-7}$ was blocked by the CXCR4 inhibitor AMD3100 and not by two CCR5 inhibitors (TAK-779 and AD-101). The opposite result was obtained with SIV$_{mac239}$, which was blocked by the two CCR5 inhibitors and not by AMD3100 (Fig. 5B). The latter result is in agreement with a previous report showing that CCR5 is the coreceptor used by SIV$_{mac239}$ for infection of macaque PBMC (26). SHIV$_{DH12R-CL-7}$ also appears to be exclusively T cell tropic since it was unable to establish spreading infections in alveolar macrophage (13).

Conclusions. A molecular clone, designated SHIV$_{DH12R-CL-7}$, has been obtained that consistently causes rapid, complete, and irreversible loss of CD4$^+$ T lymphocytes. This disease phenotype has been previously reported for three independently derived uncloned SHIVs (11, 14, 17) and is applicable to only one of the three full-length infectious SHIV$_{DH12R}$ clones obtained in this study. The acquisition of these unusual pathogenic characteristics appears to be multigenic: 42 amino acid substitutions, relative to the starting nonpathogenic SHIV$_{DH12}$ clone (20), distributed among several viral genes, were present in SHIV$_{DH12R-CL-7}$. In contrast to studies of the envelope glycoproteins associated with molecularly cloned SHIV$_{KB9}$ and SHIV$_{HXBc2P-3.2}$, which were reported to be the principal determinants inducing CD4$^+$ T-lymphocyte depletion (4, 15), the entire *env* gene of SHIV$_{DH12R-CL-7}$, by itself, failed to confer the rapid and irreversible CD4$^+$ T-cell-depleting properties following its insertion into the genome of nonpathogenic parental strain SHIV$_{DH12}$. Similarly, substitution of *gag-pol* sequences from pathogenic SIV$_{E543}$ could not replace analogous SIV$_{mac239}$ genes in SHIV$_{DH12R-CL-7}$. This result implies that the 10 amino acid substitutions introduced into the SIV$_{mac239}$ *gag* and *pol* genes (Fig. 3) also contributed to the rapid CD4$^+$ T-lymphocyte-depleting phenotype. In both cases, peak levels of plasma viremia were reduced compared to those caused by SHIV$_{DH12R-CL-7}$ and the capacity of each virus to induce CD4$^+$ T-cell depletion was minimal (Fig. 4). In the studies described here, our working definition of SHIV pathogenicity was rapid, irreversible, and complete elimination of CD4$^+$ T lymphocytes coupled with high and sustained levels of viral RNA in plasma. SHIV$_{DH12R-CL-7}$ was the only cloned SHIV that fulfilled these criteria.

The mechanism(s) underlying the rapid elimination of CD4$^+$ T lymphocytes and induction of immunodeficiency by pathogenic SHIVs remains unknown, although the increased fusogenicity of the envelope glycoprotein and the infection of a substantial fraction of CD4$^+$ T cells in lymphoid tissue during the first weeks postinoculation have been proposed to explain the unusual disease phenotype (4, 10, 15). Because the fraction of CD4$^+$ T cells in blood and lymphoid tissues expressing CXCR4 is very large (>80%) (23, 25) (Y. Nishimura and M. Martin, unpublished data), complete and systemic elimination of this T-lymphocyte subset by X4-tropic SHIV$_{DH12R-CL-7}$ could simply reflect the targeting and unrelenting depletion of these cells. It is also possible that a threshold level of systemic virus production must be reached during the first week of infection to cause the overwhelming and systemic killing of virtually all CD4$^+$ T-lymphocyte subsets in lymphoid tissues. A delay of only a few days in exceeding such a threshold, such as that due to infections initiated with low-dose virus inocula, may permit the development of immune responses capable of controlling virus replication. However, monkeys inoculated with large amounts of SHIV$_{DH12R-CL-8}$ generated peak levels of plasma viremia that were similar to those seen in animals infected with SHIV$_{DH12R-CL-7}$ (Fig. 2B and D), yet the CD4$^+$

T-cell depletion in $SHIV_{DH12R\text{-}CL\text{-}8}$-inoculated macaques was incomplete and transient (compare Fig. 2A and C). Assuming that the concentration of virus particles circulating in the blood is a reflection of SHIV replication systemically, the level of progeny virion production, per se, during the acute infection cannot be the principal determinant causing the unrelenting and complete elimination of $CD4^+$ T lymphocytes. Since $SHIV_{DH12R\text{-}CL\text{-}7}$ and $SHIV_{DH12R\text{-}CL\text{-}8}$ differ by only nine amino acids, it is likely that one or more of these residues contribute to the signature SHIV disease. Chimeric SHIVs containing individual amino acid substitutions or combinations of these amino acid substitutions are currently being constructed for inoculation into rhesus monkeys to ascertain which viral gene(s) confers the rapid $CD4^+$ T-cell-depleting phenotype.

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